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ORIGINAL RESEARCH PAPER

Engineering of geranylgeranyl pyrophosphate synthase levels and physiological conditions for enhanced carotenoid and astaxanthin synthesis in *Xanthophyllomyces dendrorhous*

Jürgen Breitenbach · Hans Visser · Jan C. Verdoes · Albert J. J. van Ooyen · Gerhard Sandmann

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Abstract The basidiomycetous yeast, *Xanthophyllomyces dendrorhous,* is one of the very few organisms which can be used for biological production of the carotenoid astaxanthin. *crtE* cDNA has been cloned from this fungus for engineering of the terpenoid pathway. The function of its gene product as a geranylgeranyl pyrophosphate synthase was established. *X. dendrorhous* was transformed with the *crtE* cDNA to divert metabolite flow from the sterol pathway towards carotenoid biosynthesis. Transformants were obtained with increased levels of geranylgeranyl pyrophosphate synthase leading to higher carotenoid levels including astaxanthin. Physiological conditions for maximum carotenoid synthesis for wild type and the CrtE transformant were dim light and extra air supply of the shaking culture. These conditions and the transformation with *crtE* had additive effects and resulted in an 8-fold higher astaxanthin formation as compared to the initial wild type culture without illumination and extra air supply yielding 451 μg/g dry wt within 4 days of growth.

Keywords Carotenoid biosynthesis · 3-hydroxy-4-keto-torulene · Geranylgeranyl pyrophosphate synthase · Light enhancement

J. C. Verdoes—deceased.

J. Breitenbach · G. Sandmann (✉)
Molecular Biosciences 213, J.W. Goethe Universität, P.O. Box 111932, 60054 Frankfurt, Germany
e-mail: sandmann@em.uni-frankfurt.de

J. C. Verdoes
Division of Industrial Microbiology, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

H. Visser · A. J. J. van Ooyen
Section of Fungal Genomics, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands

Present Address:
H. Visser
Dyadic Netherlands, Nieuwe Kanaal 7-s, 6709 PA Wageningen, The Netherlands

Introduction

Astaxanthin (3,3′-hydroxy-4,4′-diketo-β-carotene) is an industrially important carotenoid. It is used as feed additive in salmon and trout farming (Bjerkeng 2008) and has beneficial effects on human health (Hussein et al. 2006). The basidiomycetous yeast, *Xanthophyllomyces dendrorhous* (the sexual state of *Phaffia rhodozyma*), is the only fungus which synthesizes substantial amounts of astaxanthin (Andrewes and Starr 1976). Its carotenoid pathway is well established. The major end product is astaxanthin. However, a second product is 3-hydroxy-4-keto-torulene (Vazquez and Santos 1998). This keto carotenoid is formed as a by-product (see Fig. 1 for details). Only three genes cover the whole specific carotenoid

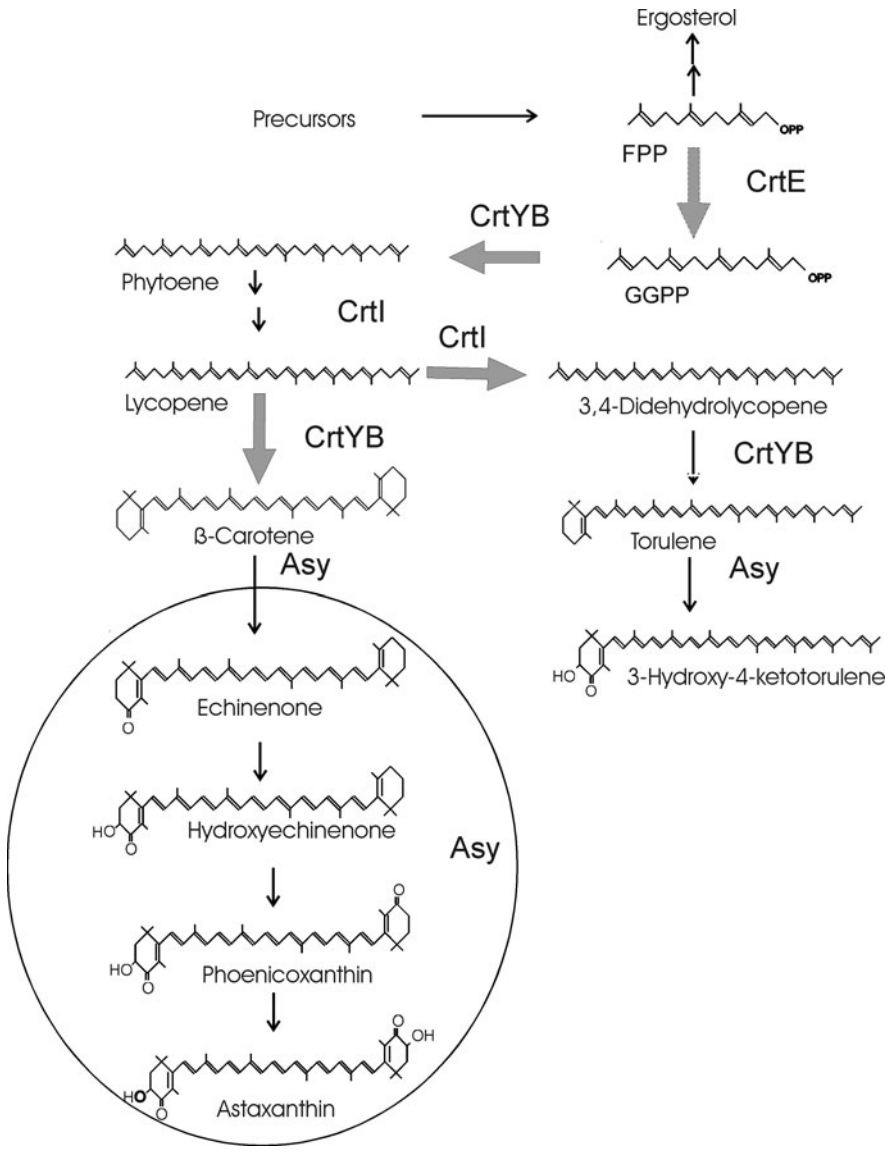

Fig. 1 Carotenogenic pathway of *X. dendrorhous* and sites (*bold arrows*) for metabolic engineering of astaxanthin synthesis. The gene products catalyzing the individual reactions are indicated: *CrtE* geranylgeranyl pyrophosphate synthase; *CrtYB* phytoene synthase/lycopene cyclase; *CrtI* phytoene desaturase; *Asy* astaxanthin synthase. All final reactions in the oval are catalyzed by astaxanthin synthase. *FPP* farnesyl pyrophosphate; *GGPP* geranylgeranyl pyrophosphate

pathway from phytoene synthesis to the end products. These are *crtYB*, a fusion gene encoding a phytoene synthase and a lycopene cyclase (Verdoes et al. 1999a), *crtI* encoding a phytoene desaturase which forms lycopene and 3,4-didehydrolycopene (Verdoes et al. 1999b) and *asy* encoding an astaxanthin synthase which converts β-carotene via echineneone (4-keto-β-carotene), 3-hydroxy4-keto-β-carotene and phoenicoxanthin (3-hydroxy-4,4′-diketo-β-carotene to astaxanthin (Ojima et al. 2006). The function of the latter enzyme is dependent on a specific astaxanthin synthase reductase (Alcaino et al. 2008).

An alternative or complementary approach to random mutagenesis for high yield strains (An et al. 1989) is genetic engineering of the astaxanthin synthesis pathway. In general, genetic modification may increase the flow of precursors into a specific pathway, mediate an efficient conversion of intermediates into the end product and divert a pathway at a branch point much stronger into a desired direction.

First attempts have been made for successful genetic engineering of the carotenoid pathway of *X. dendrorhous*. Introduction of extra copies of the *crtYB* fusion gene resulted in transformants with

higher total carotenoid content (Verdoes et al. 2003). This is due to the additional phytoene synthase activity. A second effect observed in the *crtYB* transformants was a change of the carotenoid composition with a strong decrease of 3-hydroxy-4-ketotorulene, the alternative product of *X. dendrorhous* carotenoid biosynthesis. This can be attributed to the lycopene cyclase part of *crtYB* gene product.

Feeding of the terpenoid precursor mevalonate to *X. dendrorhous* resulted in a 4-fold increase of astaxantin content (Calo et al. 1995). Consequently, we regarded synthesis of GGPP as a substrate for phytoene as a limiting step for carotenoid synthesis as found before for *Saccharomyces cerevisiae* (Verwaal et al. 2007). Therefore, we engineered *X. dendrorhous* by over-expressing its own GGPP synthase gene. The resulting increase of astaxanthin formation was analyzed under different physiological conditions i.e. by variation of O_2 supply and illumination.

Materials and methods

Strains, growth and physiological conditions

Xanthophyllomyces dendrorhous strain CBS6938 (=ATCC96594) was grown in 150 ml of a medium containing Bacto-peptone (5 g/l), yeast extract (3 g/l), malt extract (3 g/l), and glucose (10 g/l) in 1 l conical flasks at 20°C on a rotary shaker for 4 days in darkness. Selection of transformants for G418 resistance was with geneticin (G-418 sulfate, 40 μg/ml) containing agar plates. Light cultures were illuminated with low white light of 1 μmol photons per m/s. High O_2 cultures were supplemented with extra air at 50 ml/min. Transformation of *X. dendrorhous* was carried out by electroporation according to Visser et al. (2005).

E. coli strains DH5α, XL1-Blue MFR and JM101 used for genetic manipulations or functional complementation, respectively, were grown on LB medium with ampicillin (100 μg/ml) and chloramphenicol (34 mg/l).

Cloning of the GGPP synthase cDNA and construction of plasmids for *E. coli* and *X. dendrorhous*

A cDNA library from *X. dendrorhous* (Ojima et al. 2006) in *E. coli* XL1-Blue MFR was screened for a GGPP synthase cDNA by functional complementation and color selection. For this purpose, the library was co-transformed with plasmid pACCAR25ΔcrtE (Sandmann et al. 1993), which contains all the genes for the synthesis of zeaxanthin glucosides except the gene for GGPP synthase *crtE*. Functional complementation with a GGPP synthase cDNA should enable the synthesis of colored carotenoids. An orange pigmented colony was found among 8,000 white ones. This *X. dendrorhous* cDNA was used as template for further plasmids. Plasmid pUC8-2crtE was designed for *crtE* expression in *E. coli*. It was constructed by PCR amplification with primers EcocrtEforw 5′-GAATTCATGGATTACGCGAACATCCTC-3′ and crtErew 5′-GTCGACTCACAGAGGGATATCGGCTAG-3′, and sub-cloned into T-overhang vector pMON38201 (Borovkov and Rivkin 1997). The *crtE* insert was subsequently excised with *Eco*RI and *Pst*I and finally ligated into pUC8-2 (Hanna et al. 1984). The plasmid with the *crtE* cDNA for transformation of *X. dendrorhous* was obtained by amplification with the primers PcrtEATG 5′-ATATGAATTCATGGATTACGCGAACATCCTC-3′ and PcrtETGA 5′-TATAGTCGACTCACAGAGGGATATCGGCTAG-3′ which generated *Eco*RI and *Sal*I cloning sites. The obtained PCR product of expected size (1.1 kb) was ligated into the *Eco*RI and *Xho*I sites of vector pPRcDNA1 (Ojima et al. 2006) yielding pPRcDNA1crtE.

Carotenoid extraction and analysis

Cells of *X. dendrorhous* were harvested and freeze-dried. Carotenoids were subsequently extracted from these cells with dimethylsulfoxide by heating for 15 min at 60°C. After centrifugation, the extract was transferred into a separator funnel, diethyl ether was added, and the mixture was kept on ice for 3 min before water was added for phase separation. The upper phase was collected to which step by step acetone, 10% (v/v) diethyl ether in petroleum ether (bp 60–80°C) and water were added. Again, the upper phase was collected, washed with water, dried in a stream of N_2, and re-suspended in acetone. Details of the extraction procedure are given elsewhere (Visser et al. 2005).

Freeze-dried *E. coli* cells were extracted with methanol by heating for 15 min at 60°C. The extract was partitioned against 50% (v/v) ether in petrol and the upper phase collected and dried. High-performance liquid chromatography (HPLC) separation of

carotenoids was performed on a 25 cm HyPurity C18, 5 μ column with acetonitrile/methanol/2-propanol (85:10:5, by vol) as the mobile phase. Spectra were recorded on-line with a photodiode array detector. Carotenoid identification and quantitation was carried out with authentic standards.

Results and discussion

Function of *crtE* and the *X. dendrorhous crtE* transformation vector

The cloned GGPP synthase cDNA *crtE* from *X. dendrorhous* encoded a protein of 375 amino acids. The sequence of the cDNA and genomic DNA is presented elsewhere (van Ooijen et al. 2007). The function of the cDNA was determined by pathway complementation in *E. coli* using plasmid pAC-CAR25ΔcrtE, which contained all genes for the synthesis of zeaxanthin diglucoside except the GGPP synthase gene (Sandmann et al. 1993). Upon separation by HPLC, only minor amounts of zeaxanthin diglucoside and zeaxanthin of together 4 μg/g dw were detectable due to the very low GGPP pool in *E. coli*. When *E. coli* was co-transformed with pUC8-2crtE (for vector map see Fig. 2 top), which was designed for *crtE* expression, the concentration of the same carotenoids including small amounts of zeaxanthin monoglucoside increased more than 30-fold to 128 μg/g dw. This result indicated that the CrtE from *X. dendrorhous* indeed is the GGPP synthase as shown in Fig. 1.

After assignment of the *crtE* cDNA as a GGPP synthase, it was used to construct a transformation plasmid for *crtE* genome integration and over-expression in *X. dendrorhous*. The resulting plasmid is shown in Fig. 2 bottom. Both the *crtE* cDNA and the *nptII* gene conferring resistance of transformed *X. dendrorhous* to geneticin (G418) were flanked by the promoter and terminator of the *X. dendrorhous* glyceraldehyde-3-phosphate dehydrogenase gene. The *crtE* transformation vector contained a ribosomal DNA sequence region for multiple copy integration into the *X. dendrorhous* genome. Integration of the transformation vector was achieved after linearization by the single-cutting restriction enzyme *Sfi*I prior to transformation.

Carotenoid formation in *X. dendrorhous* CBS6938[crtE] transformants

Since after transformation of *X. dendrorhous*, variations in the resulting phenotype can be found depending on the number of integrated copies (Verdoes et al. 2003), different CBS6938[crtE] transformants were selected according to the intensity of their pigmentation. The carotenoids of the three most pigmented transformants are shown in Table 1. They synthesize between 1.3- and 1.5-fold more total carotenoids than the control reaching up to 288 μg/g dry weight. Transcript determinations of *crtE* in transformant no. 1 revealed a 10 times higher level than in the wild type strain (data not shown). In spite of the resulting enhanced overall carotenoid synthesis, formation of the pathway end product astaxanthin did not increase accordingly. Instead, also keto carotenoid intermediates accumulate. This result indicates that the transformants have a certain limitation in the biosynthesis pathway to astaxanthin for the last steps catalyzed by Asy (Fig. 1). A similar observation was also reported for *X. dendrorhous* transformants that over-expressed the *crtYB* gene (Verdoes et al. 2003).

The major terpenoid in fungi is ergosterol. Its synthesis branches off the route to carotenoids at the level of farnesyl pyrophosphate, the substrate for GGPP synthase. The concentrations of ergosterol and carotenoids including colorless phytoene were determined in wild type CBS6938 and the transformant CBS6938[crtE] with over-expressed GGPP synthase (Table 2). In the wild type strain grown without extra air supply, the carotenoid content was substantially lower than ergosterol regardless whether permanently illuminated or not. Enhanced air supply increased colored carotenoids at the expense of ergosterol. Additionally, the phytoene content decreased. These results suggest a better utilization of FPP towards carotenogenesis and a more efficient conversion within the carotenoid pathway towards the end products. This up-regulation of astaxanthin synthesis with higher O_2 supply may be due to generated singlet oxygen and oxygen radicals (Schroeder and Johnson 1995). Another environmental factor which can stimulate carotenogenesis in *X. dendrorhous* is light (de la Fuente et al. 2010). With our wild type strain, the highest astaxanthin and 3-hydroxy-4-ketotorulene concentrations were obtained in the light with extra air supply, which with 452 μg/d dry weight were

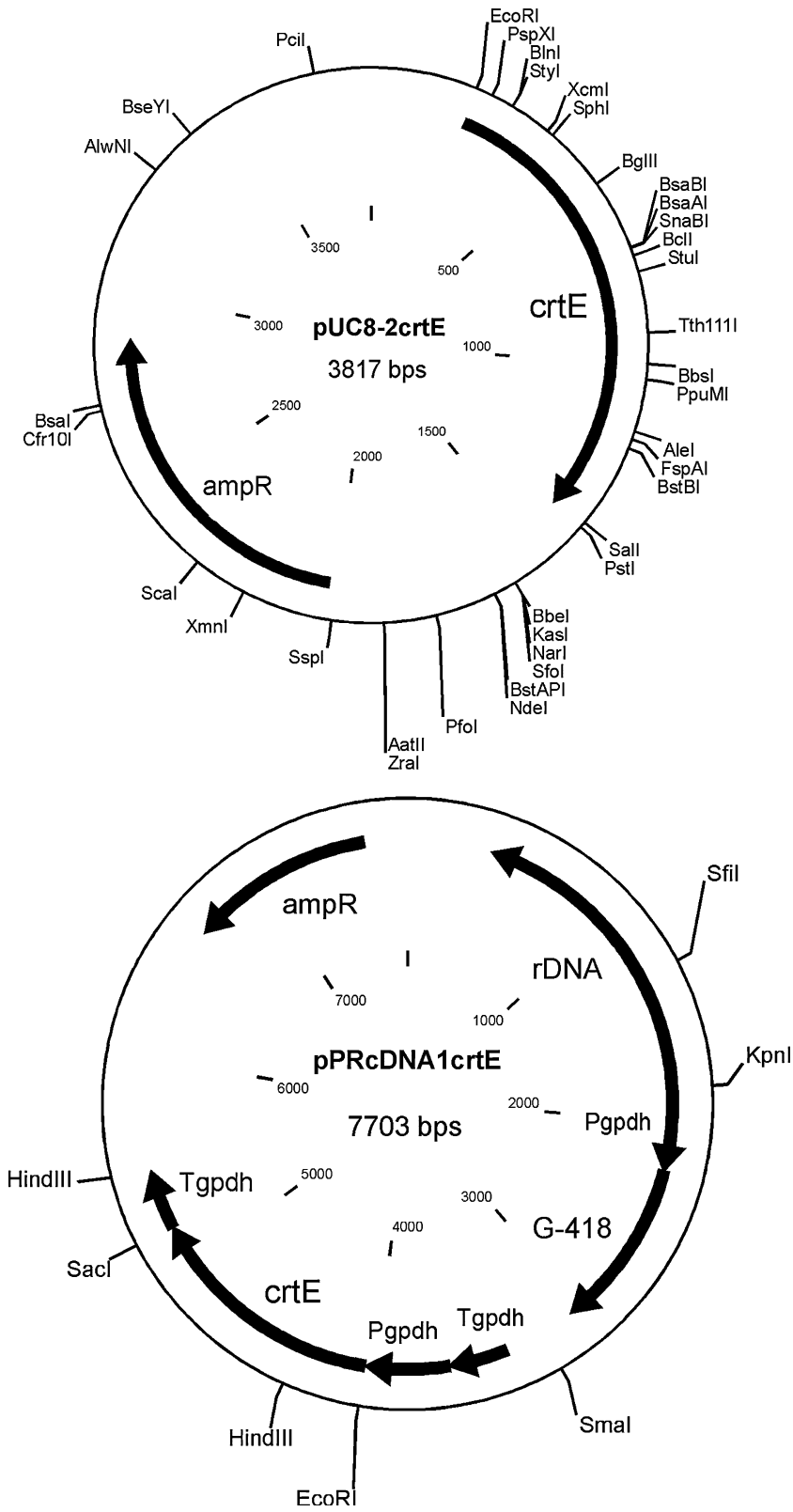

Fig. 2 Transformation vectors pUC8-2crtE for CrtE expression in *E. coli* (*top*) and pPRcDNA1crtE for over-expression of geranylgeranyl pyrophosphate synthase in *X. dendrorhous* (*bottom*)

Table 1 Total colored carotenoids and % distribution of individual carotenoids in *Xanthophyllomyces dendrorhous* wild type CBS6938 (control) and individual *crtE* transformants CBS6938[crtE] (numbers 1, 2, 3)

	Control	1	2	3
Carotenoids (μg/g dw)	198 ± 38	288 ± 56	263 ± 50	259 ± 57
Distribution (%)				
Astaxanthin	37.5	29.1	32.4	28.6
Hydroxycanthaxanthin	13.8	12.8	18.0	19.4
Hydroxyketotorulene	15.7	27.7	21.2	19.5
Hydroxyechinenone	11.3	12.1	6.1	13.4
Echinenone	8.7	5.3	7.4	8.3
Lycopene	7.0	0	2.2	0
Torulene	0.5	1.6	3.8	4.0
β-Carotene	6.5	11.4	8.8	6.8

Growth was for 4 days without extra air supply, in the dark at 20°C as shaking cultures. Means ± standard deviation were from 3 to 5 determinations

Table 2 Ergosterol and carotenoid formation (μg/g dw) in wild type *X. dendrorhous* CBS6938 and a transformant with integrated *crtE* gene CBS6938[crtE]#1

Conditions	Ergosterol	Phytoene	TCC	3HO4KT	Astaxanthin
CBS6938					
Dark, LO	1060 ± 257	84 ± 13	167 ± 11	16 ± 7	57 ± 8
Dark, HO	864 ± 342	5 ± 3	602 ± 28	53 ± 9	147 ± 11
Light, LO	1014 ± 208	57 ± 5	223 ± 17	20 ± 4	56 ± 8
Light, HO	975 ± 333	10 ± 7	694 ± 49	87 ± 12	304 ± 29
CBS6938[crtE]					
Dark, LO	1042 ± 554	104 ± 20	244 ± 18	0.6 ± 7	77 ± 19.6
Dark, HO	881 ± 206	50. ± 6	963 ± 54	106 ± 14	250 ± 32
Light, LO	1029 ± 164	98 ± 11	369 ± 43	20 ± 8	111 ± 8
Light, HO	916 ± 104	40 ± 15	1007 ± 65	126 ± 13	452 ± 44

LO low O_2 without extra air supply; *HO* high O_2 with extra air supply; *TCC* total colored carotenoids; *3HO4KT* 3-Hydroxy-4-keto-torulene; means ± standard deviation from 3 to 5 determinations

5-fold higher than in the dark, culture without extra air. The same tendency was observed for the *crtE* transformant. Here, we obtained an additive effect between GGPP synthase over-expression, illumination and O_2 supply, which all contributed to increased synthesis of total carotenoids including astaxanthin. In the case of astaxanthin, the yield was 8-fold higher than in the initial wild type culture without illumination and extra air supply reaching a maximum of 451 μg/g dry wt.

Conclusion

Over-expression of *crtE* is a useful target to divert and enhance metabolite flow into carotenogenesis. In addition, physiological conditions like dim light illumination of and enhanced air supply to the shaking culture all independently contributed to enhanced astaxanthin synthesis in an additive way. Although astaxanthin yield could be strongly increased, its amount accounts only for 43% of total carotenoids even under optimum conditions. Most of the residual carotenoids are keto derivatives which are not completely metabolized into astaxanthin. This finding suggests that *asy* over-expression should be the next step to increase astaxanthin yield. Previous transformation with additional copies of the *crtYB* gene was already successful in increasing carotenoid formation (Verdoes et al. 2003). Therefore, maximum astaxanthin biosynthesis can finally be achieved both by

generating a fully genetically engineered *X. dendrorhous* with additionally expressed *crtE*, *crtYB* and *asy* genes which are likely to alleviate limitations of the carotenoid pathway in combination with advantageous cultivation conditions. This overall genetic approach will represent a decisive step towards the development of *X. dendrorhous* as a cell factory for astaxanthin production which may economically compete with chemical synthesis.

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